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The Degradation of Semisynthetic Tritiated Insulin by Perfused Mouse Livers

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Semisynthetic [^{3}H]insulin was stored under various conditions for up to 180 days and the stability of the insulin under these conditions was assessed. A sample that had been stored for 180 days was repurified and shown to be degraded at the same rate as native insulin by perfused mouse livers, even at low physiological concentrations. After perfusion, intact insulin could be separated from degradation products, and the radioactivity associated with the insulin fraction could be used to determine the percentage degradation. The initial rate of degradation of insulin was a linear function of concentration over the range 360pM–1.9nM.

Studies on the metabolism of insulin call for a radioactively labelled analogue of the hormone. The specific radioactivity should be high enough to make it possible to detect the products of insulin degradation, and yet the analogue must be unaltered in its biological behaviour. Semisynthetic [^{3}H]insulin has been prepared by the specific removal of the *N*-terminal amino acid of the insulin B-chain (phenylalanine) and its replacement with [^{3}H]phenylalanine (Halban & Offord, 1975). Because of the minimal alteration of the insulin caused by the isotopic substitution, it would be expected that the ^{3}H-labelled analogue would be more authentic in its properties than any radioiodinated insulin, the labelled insulin most commonly used. It has been shown (Halban & Offord, 1975) that the [^{3}H]insulin was indistinguishable from native insulin in its hypoglycaemic effect on mice, and that it was chemically homogeneous. By contrast, radioiodinated insulins often show alterations in behaviour compared with the native hormone, either as a result of chemical damage during the iodination or because of the addition of a bulky iodine atom. Experiments on the rate of metabolic clearance of insulin by intact animals or perfused organs exemplify the problems involved. Some of the studies where a comparison has been made between the rate of clearance of native insulin (determined by immunoassay) and radioiodinated insulin (determined by radioactivity) have produced conflicting results. In the majority, the labelled hormone was cleared markedly more slowly than was the native hormone [Genuth (1972), and compare Stern *et al.* (1968) with Shen *et al.* (1970)]. On the other hand Terris & Steiner (1976) found close similarities in the behaviour of various mixtures of ^{125}I-labelled and native insulin, although they used total concentrations in the range 10nM–5μM.

The present paper reports a comparison between the rate of clearance of [^{3}H]insulin with that of native insulin in the concentration range 360pM–1.9nM. No significant difference could be found. We have also carried out experiments that establish the conditions for long-term storage of the ^{3}H-labelled material. Because of the relatively short half-lives of the radioisotopes involved, the question of long-term storage of radioiodinated insulins has never arisen. We have evaluated two simple methods for removing any denaturated material before use.

Materials and Methods

Semisynthetic [*phenylalanine*^{B1}-^{3}H]insulin (specific radioactivity 15Ci/mmol) was prepared as previously described (Halban & Offord, 1975). [In that paper the concentration of protein in steps (iv) and (v) was given, in error, as 6ng/μl; the correct value is 6μg/μl.] The radioactivity of the tritiated sample was determined in a Beckman liquid-scintillation counter (model LS-230) by using Instagel (Packard Instrument International S.A., Zurich, Switzerland) as the scintillant. Immunoassays were performed by the method of Herbert *et al.* (1965), by using antiserum to pig insulin generously provided by Dr. Peter Wright (Indiana University School of Medicine, Indianapolis, IN, U.S.A.). Bovine serum albumin for the liver perfusions, Sephadex G-50 column chromatography and immunoassays was supplied by Behringwerke A.G., Marburg/Lahn, West Germany, and defatted by the method of Chen (1967). Bovine serum albumin for the affinity-column chromatography was a

Pentex-grade product from Miles Laboratories, Kankakee, IL, U.S.A. Sephadex G-50 was a Pharmacia product, supplied by Instrumenten Gesellschaft, Geneva, Switzerland. CNBr-activated Sepharose 4B for affinity-column chromatography [Pharmacia (G.B.) Ltd., London W.5, U.K.] was treated according to the manufacturer's instructions to bind covalently anti-insulin serum (Burroughs Wellcome, Dartford, Kent, U.K.); 0.01 ml of serum was used for 0.03 g dry wt. of activated Sepharose. Binding of insulin to the affinity column (0.3 cm × 1.5 cm) and the subsequent elution of the bound material with acetic acid was carried out by the method of Akanuma *et al.* (1970). The eluted material was dialysed to remove the acid before immunoassay. Lean mice (C57 BL/6J) bred in our Geneva laboratories were used for perfusion studies. Mice were anaesthetized by intraperitoneal injection of phenobarbital (Nembutal, supplied by Abbott A.G., Zug, Switzerland) at 50 mg/kg body wt. The livers were perfused *in situ* by the method of Assimacopoulos-Jeannet *et al.* (1973) as applied to the study of hepatic insulin clearance by Karakash *et al.* (1976). A recycling perfusion system was used. The perfusion medium was a Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 3% (w/v) defatted bovine serum albumin and 30% (v/v) washed bovine erythrocytes. The medium was recirculated for 1 h and 3 ml samples were removed at 0, 10, 20, 30, 45 and 60 min. Perfusions were carried out at 37°C and the flow rate was 3 ml/min. After perfusion, the livers were removed and weighed. Samples of the perfusate were kept on ice before centrifugation to remove erythrocytes (2000*g* for 10 min). Perfusate samples and native pig insulin for the standard curve (Novo Terapeutisk Laboratorium, Bagsvaerd, Denmark) were diluted in Krebs–Ringer bicarbonate buffer containing 3% bovine serum albumin for the radioimmunoassay. The results of the immunoassay carried out on the perfusate supernatants were corrected for the decrease in the total volume of the perfusion medium caused by sample removal. Since the total initial volume was 60 ml and six 3 ml samples were removed during the 1 h perfusion, this effect was significant.

Results and Discussion

Storage and purification of [^{3}H]insulin

Whereas most batches of freshly prepared semisynthetic [^{3}H]insulin are found to be fully biologically active after only one purification step on Sephadex G-25 (Halban & Offord, 1975), some batches have been found to contain tritiated non-immunoreactive material and to require a further purification step. Two separation techniques were examined for this purpose. The first was the immunoaffinity column described in the Materials and Methods section. The capacity of the column for insulin, as determined by a Scatchard plot, was approx. 0.05 μg/ml of swollen adsorbent. The same plot indicated a dissociation constant of binding of approx. 10^{-7} M. That the non-immunoreactive material had been successfully removed by this method was shown by the fact that the unretarded material contained 50 times as much radioactivity per ng of immunoreactive insulin as did the bound and eluted material. The specific radioactivity of the bound and reluted material was approximately that expected of fully immunoreactive material.

The second technique was to pass the material through a Sephadex G-50 column (0.8 cm × 60 cm) by using 0.2 M-glycine/0.25% bovine serum albumin, adjusted to pH 8.8 with 5 M-NaOH, as the elution buffer. Three radioactive peaks were eluted from the column. The first, which was eluted at the position of albumin, probably consisted of inactive insulin associated with albumin. The second, and major, component was eluted with insulin and was found to be fully immunoprecipitable. The third peak contained molecules smaller than insulin. The specific radioactivity of the pooled insulin fractions was found to be the same as that of the fraction bound and subsequently eluted from the immunoaffinity column. Because of its relative ease of use, the Sephadex column is recommended for purification of [^{3}H]-insulin, but it cannot be predicted that it would be as satisfactory as the immuno affinity column for all semisynthetic analogues of insulin.

In order to be able to take advantage of the relatively long half-life of ^{3}H, it is essential to establish satisfactory conditions for the long-term storage of the [^{3}H]insulin with only minor losses of biological activity, and to be able to separate any inactive material from fully active insulin. We have stored identical samples under several conditions (frozen at −20°C, frozen under liquid N_2, freeze-dried and stored under liquid N_2 and on a 0.1 ml immunoaffinity column at 4°C in the buffer described above) for 9, 27 and 80 days. In addition, samples frozen at −20°C were stored for 180 days. The affinity column was used to determine the fraction that remained able to bind to antiserum at the end of the storage period. For all methods of storage below 0°C there seemed to be an immediate loss of 20–30% of the binding potential, and little or no progressive loss thereafter. The material stored on the affinity column showed a 50% loss of binding potential after 80 days. The material stored at −20°C for 180 days was also examined by the Sephadex method and it was found that 80% of the radioactivity was associated with the active-insulin peak. The material recovered from the insulin peak in this way was fully immunoprecipitable and indistinguishable from native insulin in its hypoglycaemic effect on mice.

Degradation of stored and purified [^3H]insulin by perfused mouse livers

[^{3}H]Insulin (stored for 180 days at −20°C) was purified by Sephadex G-50 chromatography as described above. The rate of degradation of the purified product by perfused mouse livers was compared at concentrations in the physiological range with that of native insulin and a 1:1 mixture of native and [^{3}H]insulin. Samples of the perfusate were assayed for immunoreactive insulin after removal of erythrocytes by centrifugation (see the Materials and Methods section). Each sample was assayed in triplicate at two different dilutions. Starting concentrations of insulin in the range 2.1–11 ng/ml (360 pM–1.9 nM) were used. Three mice were used for each starting concentration of a given insulin preparation and samples were removed at six times during 1 h perfusion. The initial rates of degradation (after analysis of the insulin decay curves by a polynomial regression) for each starting concentration were calculated and plotted against the starting concentration. A straight line was obtained (Fig. 1), which indicates that the system was not saturated in the range of insulin concentrations used. Further, all three insulin solutions gave results which lay on the same line, indicating no significant difference between native and tritiated insulin. Despite the linear relationship between the initial rate of degradation and the starting concentration, it was found that the observed rate of degradation during the recycling perfusions decreased more rapidly with time than would have been predicted by this relationship. After 1 h of perfusion, the decay curves appeared to level off almost completely at all the concentrations studied. This might be an indication of a competitive effect caused by the accumulation of degradation products. Control experiments (in the absence of livers) showed no significant degradation of either tritiated or native insulin after 1 h incubation in the perfusion chambers. The radioactivity associated with the erythrocyte pellet was found to be less than 3% of that of the supernatant and did not change with the length of perfusion time. The liver was found to retain less than 5% of the radioactivity.

Samples of supernatants from the perfusions with [^{3}H]insulin were also passed down a Sephadex G-50 column (under the same conditions as those described for the purification step above). Whereas the percentage of radioactivity eluted with the albumin fraction remained constant throughout the perfusion, that eluted with the insulin fraction decreased with time as the percentage of small molecules increased. A similar technique was used for iodinated insulin by Terris & Steiner (1976). Since it has been shown

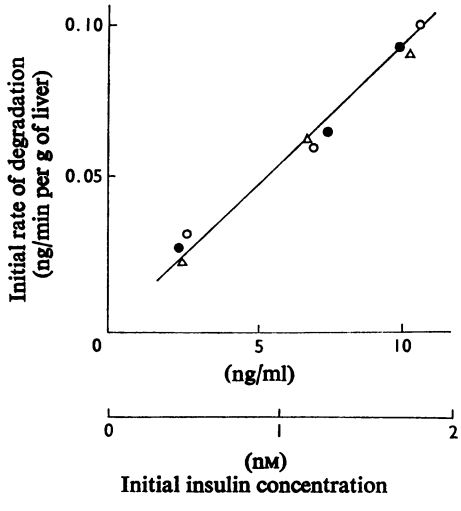

Fig. 1. *Plot of the initial rate of insulin degradation against the starting concentration*

The initial rate of degradation (ng/min per g of liver) was derived from the initial slope of the regression plots in insulin decay (determined as described in the text). The mean of the three independent observations at each sample time was plotted against time to give an insulin decay curve. The S.E.M. of each point on the decay curve was less than 5%. A correlation coefficient of 0.982 was obtained by linear regression ($P<0.001$). △, [^{3}H]Insulin; ●, [^{3}H]-insulin+native insulin (1:1); ○, native insulin.

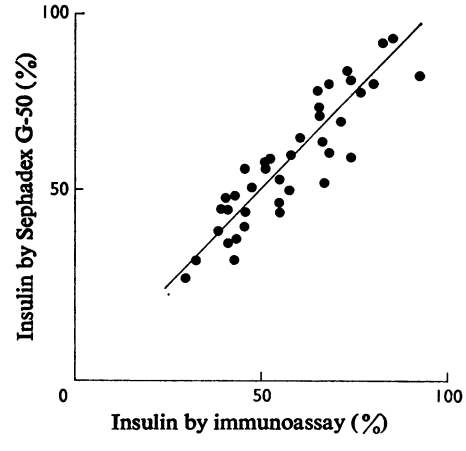

Fig. 2. *Comparison of the determination of insulin by the Sephadex G-50 method and by immunoassay*

The Sephadex G-50 method is described in the text. The units represent the content of insulin as a percentage of the starting concentration determined at various times during the perfusion. A correlation coefficient of 0.926 was obtained by linear regression ($P<0.001$), $n=39$.

above that the insulin fraction eluted from the Sephadex column was fully immunoprecipitable, it was thought that the percentage of radioactivity associated with the insulin fraction after perfusion might be an independent means of assessing the percentage degradation of the [^{3}H]insulin and, so long as the starting concentration was known, of determining the remaining concentration of intact insulin at any time. Fig. 2 shows a comparison of the percentage of insulin calculated by this method and by immunoassay; there is a close correlation between the two methods. The availability of two independent assay methods would make it possible to compare, for example, the fate of two insulin samples (one of which would be tritiated) in the same perfusion system or even *in vivo*.

A preliminary examination of the ^{3}H-labelled material of lower apparent molecular weight than insulin generated during the perfusion suggests that it should be possible to determine the chemical nature of the fragments. A similar approach has been used by Varandani & Nafz (1976) to study the degradation of iodinated insulin by isolated liver cells. Our work should be assisted by the fact that, in contrast with many preparations of radioiodinated insulin, the precise location of the label on the [^{3}H]-insulin is known.

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